Dominant bacteria associated with broods of mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Curculionidae, Scolytinae)

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ABSTRACT

Mountain pine beetle (MPB) is the most damaging insect of mature pine forests in western North America. The current outbreak in British Columbia is the largest ever recorded. During a survey of beetle occurrence, well-established infestations were sampled in central B.C. and found to possess larval mortality. Bacteria or other microbes were among the potential causes of the mortality. Bacteria were isolated from living larvae and adults, as well as larval and adult beetle cadavers found in bark samples. Bacteria were identified by fatty acid methyl ester (FAME) analysis, which indicated 32 species of bacteria present in the MPB larvae. The predominant bacteria (*Serratia liquefaciens*, *S. plymuthica*) were detected in about a third of all sampled larvae, regardless of mortality. *Rahnella aquatilis* was found in 11% of all larvae examined and was usually (93%) associated with larval mortality. Interactions between two bluestaining fungal symbionts of the MPB (*Grosmannia clavigera*, *Ophiostoma . montium*) and two of the isolated bacteria (*S. liquefaciens* and *R. aquatilis*) were assessed. *S. liquefaciens* and *R. aquatilis* both inhibited the growth of beetle-associated bluestain fungi by 72%. The bluestain fungi did not impede bacterial growth, and both bacteria grew on autoclaved bluestain mycelium. Combinations of the two bacterial species formed aggregates on practical-grade (crab) chitin, but there was no aggregation in pure cultures or on the autoclaved mycelium of *G. clavigera* or *O. montium*. These results indicated that the two bacteria may be capable of aggregation within the insects, and this may have implications for their combined effects in the beetle. The role of *S. liquefaciens* and *R. aquatilis* in MPB biology requires further investigation.

Key Words: bark beetle, disease, larva, pathosystem

INTRODUCTION

As associates, antagonists, and pathogens, microbes play important roles in the life cycle of bark beetles (Barras and Perry, 1975), including the Mountain Pine Beetle (MPB), *Dendroctonus ponderosae* Hopkins. MPB has been responsible for billions of board feet of timber losses (Anonymous 2005, Bellows *et al.* 1998); a current outbreak has spread over a vast area of British Columbia (BC), killing at its peak ca. 1.41×10^8 m^3 of merchantable pine; during 2008 the outbreak was still killing 3.6-4.3 $\times 10^6$ m^3 of merchantable pine (Walton, 2009). The potential for microbial populations to constrain the beetle is therefore of potential interest. In most of BC, MPB has a 1-year life cycle, where incipient or epidemic populations attack trees *en masse*. Young female adults emerge from host trees during late summer, and initiate the attack on new host trees by burrowing into the phloem and tunnelling vertically to form egg galleries. Hatched larvae overwinter and feed in the phloem. Pathogenic

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bluestain fungi associated with the beetles spread from the galleries; eventually, trees succumb to this combined attack. (Safranyik and Carroll, 2006) During the spring of 2003, a routine survey of larval and adult MPB in central BC detected the presence of large numbers of dead larvae. The larvae were in lodgepole pine (*Pinus contorta* Dougl. ex Loud.) trees containing well-established broods. Although the patchy occurrence of very cold temperatures in the region was suspected as one possible cause for the mortality, the atypically darkened and distended hindguts of the larvae suggested that microbial factors should not be ruled-out.

Bluestain fungi are microbes that have a close relationship with the MPB life cycle; they also figure prominently in interactions with other beetle-associated microbes. MPB possesses a specialized mycangium that carries the inoculum of bluestain fungi from tree to tree (Whitney and Farris 1970). The main fungi associated with MPB are *Grosmannia clavigera* (Robinson & Davidson) Zipfel, deBeer & Wingf. and *Ophiostoma montium* (Rumbold) von Arx, and their relative abundances in populations varies (e.g., Kim et al. 2005, Six and Bentz 2007, Bleiker and Six 2007, 2009). As larval broods develop, the fungi colonize the phloem and sapwood (Whitney 1971, Bleiker and Six 2009). Mining larvae may ingest phloem colonized by hyphae and teneral adults consume spores lining pupal chambers if present prior to emergence (Whitney 1971, Bleiker and Six 2007, 2009). These fungi are reported to have mutualistic and antagonistic relationships with bacteria and yeasts found in the beetle galleries (Adams *et al.* 2008).

Natural enemies of *Dendroctonus* spp. are diverse (Bellows *et al.* 1998, Bushing 1965, Dahlsten 1982, Moore 1971, 1972a, 1972b). They play a role in holding MPB populations in check during their endemic phase (Moeck and Safranyik 1984, Hofstetter *et al.* 2006). Some natural enemies of MPB have been studied as potential biological control agents, for example the insect pathogen *Beauvaria bassiana* (Balsamo) Vuillemin (Hunt *et al.* 1984). Various options have been suggested for augmenting natural biological control effects, including the inundative release of microbes that may be antagonistic to the bluestain fungi (Safranyik et al. 2002). The objective of this research was to determine the dominant bacteria present in living and dead larvae and adults of MPB and to characterize interactions between the two principal bluestain fungi associated with MPB and the dominant bacteria associated with larvae. Our hypothesis was that different bacteria would predominate in living vs. dead insects, and that bacteria predominating in dead insects would be inhibitory to microbial associates of the living beetles, i.e. bluestain fungi and predominant bacteria.

MATERIALS AND METHODS

Isolation of Bacteria and Fungi from Beetles. During the first week of March, 2003, Rectangular 20x30-cm slabs of sapwood with the bark and phloem intact were cut with a chainsaw from five randomly-selected beetle-attacked lodgepole pine trees at each of ten sites (Table 1, Fig. 1). The slabs were taken at points 1.3 m from the ground at a randomly selected aspect (north, south, east or west). The geographic position of each site was recorded with a GPS unit, and site moisture (humid, mesic, or xeric) and the percentage of currently attacked trees in the surrounding stand were visually estimated. The slabs were stored at 20°C on a laboratory bench for several days, thus ensuring activity of living larvae. In the laboratory, the bark was peeled from the sapwood with a knife and MPB adults (parental) or larvae were excised with forceps and surface-sterilized according to the method of Winder and Watson (1994). Insect mortality was assessed in each slab as the percentage of dead larvae and adults versus the total number of insects present. Larvae were considered to be dead when

Table 1.
Descriptions of sites in British Columbia where mountain pine beetle larvae were collected in March, 2003.

Site (no.)	Location	Elevation (m)	Moisture (class)	Infested trees (%)	Terrain (type)
1	Agodak L.	1132	Hydric	50	Flat
2	Quesnel B.	1189	Hydric	60	Flat
3	5100 Rd. 1	1007	Hydric	60	Northern slope
4	5100 Rd. 2	1040	Mesic	70	Southern slope
5	Baker C 1	1009	Mesic	80	Western slope
6	Baker C 2	976	Mesic	60	Western slope
7	6600 Rd. 1	979	Mesic	60	Western slope
8	6600 Rd. 2	854	Mesic	50	Eastern slope
9	Redstone 1	900	Xeric	10	Flat
10	Redstone 2	859	Xeric	50	Flat

they appeared to have abnormal, distended hindguts and they failed to move upon firm, repeated probing with a dissecting needle. Adults were considered to be dead when they were overcome with yeasts or fungal hyphae and non-reactive to probing. Zero to three live larvae, zero to three dead larvae, and one to two adults (living or dead) were selected from each slab, using the number available to a maximum of three. The larvae and beetles were placed on nutrient agar (NA) in Petri plates; NA was composed of 8 gL^{-1} Difco Bacto® dehydrated nutrient broth and 20 gL^{-1} agar (Sigma). The insects were tamped downward with enough force to partially embed them in the medium. The plates were sealed with paraffin film and incubated at 20±2°C for 7d to allow microbial colonies to form. A colony possessing predominant morphology (colour, size, and growth pattern) was selected from each insect sample and aseptically streaked on NA plates for selection of pure cultures. The plates were sealed with paraffin film and incubated at 20±2°C for 1-2 weeks prior to identification. One pure colony possessing predominant morphology was selected per insect, for subsequent culturing according to the same method.

Identification of Bacteria. Bacteria were identified by fatty acid methyl ester (FAME) analysis using the Sherlock® Microbial Identification System (MIDI Inc., Newark, DE, USA). Pure colonies were transferred by quadrant-streaking onto plates of BBL® Trypticase Soy Broth agar (TSBA) and incubated for 24h at 28±2°C. This was repeated once again prior to analysis. Bacterial biomass was harvested from the third quadrant of the final cultures, and fatty acids were saponified and methylated according to the protocols provided by MIDI Inc. for the Sherlock® system. The resultant fatty acid methyl esters were extracted into 1:1 (volume-to-volume) hexane: methyl tertiary butyl ether (MTBE) and analyzed with a gas chromatograph (5890A Series II with HP-Ultra 2 column, Hewlett-Packard Co., Mississauga, Ont., Canada) using the TSBA40 method for aerobic bacteria, also provided by MIDI Inc. for the Sherlock® system. Extracted *Stenotrophomonas maltophilia* (Hugh) Palleroni & Bradbury (ATCC 13637) and a hexane:MTBE reagent blank were used as positive and negative controls, respectively. Bacterial isolates were identified from comparisons made against reference strain data (Anonymous, 2002) in the MIDI TSBA40 database, version 4.10 (MIDI Inc., Newark, DE, USA). Using the protocol and standards published by Weyant et al. (1996), a similarity index (SI) ≥ 0.500 was considered an acceptable identification to the level

Figure 1. Sites sampled for mountain pine beetles, with numbered locations corresponding to Table 1.

of species. SI values ≥ 0.300 were considered an acceptable identification to the level of genus, while lower similarity index values were considered inconclusive. When comparisons resulted in multiple species or genera exceeding these identity thresholds, data corresponding to the highest SI value were used for the identification. Dendrogram cluster analysis based on unweighted pair-matching of fatty acid profiles (Sherlock® analysis software, MIDI Inc., Newark, DE, USA) was used to explore relatedness among unidentified isolates.

Bacterial inhibition of Bluestain

Fungi. A nested experimental design was used to assess interactions between the three most dominant bacteria: *Serratia liquefaciens* (Grimes & Hennerty) Bascomb *et al.*, *Serratia plymuthica* (Lehmann and Neumann) Breed *et al.*, and *Rahnella aquatilis* Izard *et al.*), and two bluestain fungi commonly associated with *D. ponderosae*, *G. clavigera* and *O. montium* (Solheim and Krokene 1998). Fungal isolates were obtained from C. Breuil (University of British Columbia, Vancouver, BC) as cultures growing in Petri Dishes containing malt extract agar. Small pieces of these cultures were aseptically transferred to Petri dishes containing 3:7 (v:v) malt extract agar:NA. This substrate was optimal for simultaneous fungal and bacterial growth in preliminary trials combining different ratios of the two agar media (data not shown). To account for the variable effects of cultural moisture on microbial growth, Petri dishes were treated as experimental units, and the difference in fungal growth in the presence or absence of bacteria was evaluated in each dish. Half of each dish was inoculated with a 'lawn' of either *S. liquefaciens, S. plymuthica*, or *R. aquatilis*. In each dish, a 5 mm-diam. agar plug colonized by either *G. clavigera* or *O. montium* was placed on the bacterial side, and another was placed on the bacteria-free side. In each plate, the bacterial inoculum was taken from one of five randomly selected isolates from each of the three species of bacteria collected from the beetles. Each isolate originated from a different beetle. Each combination of fungus and bacterial isolate was replicated five times. The plates were incubated for 7 d at 20°C. The radial growth of fungal colonies was measured after incubation and percentage of growth inhibition was calculated for each plate by dividing the maximal radius of the fungal colony on the bacterial side by the maximal radius of colony in the bacteria-free area, and multiplying by 100.

Interactions between bacteria and chitin-containing substrates. A second completely randomized experiment was used to assess the interactive effect of bacteria and chitin-containing substrates on bacterial growth and aggregation, because it is possible for bacterial consortia to affect insect health (Hentzer and Givskov 2003) or ice nucleation (Pierson *et al.* 1998) and therefore cold tolerance. A sterile transfer loop was used to aseptically transfer bacteria from cultures of *R. aquatilis* and *S. liquefaciens* to sterile water (100 mL) in 250 mL Erlenmeyer flasks, and the bacterial concentration in each case was diluted to 1 x 10^6 bacteria mL^{-1} based on measurements with a Petroff-Hausser counting chamber (Hausser Scientific Partnership, Horsham PA) and a microscope (1000 X). Equal parts of the bacterial suspensions were combined to make a third combined bacterial suspension, resulting in a concentration of 0.5 x 10^6 bacteria mL^{-1} for each bacterial species. Each of the three suspensions was aseptically transferred with a sterile transfer loop to an individual set of nine sterile aqueous cultures in 250 mL Erlenmeyer flasks containing 0.1 g (d.w.) of substrate. In each culture set, the substrate in three flasks consisted of autoclaved hyphae of *G. clavigera*; autoclaved hyphae of *O. montium* was included as the substrate in three more flasks, and the three remaining flasks contained chitin derived from crab shells (poly-N-acetyl-glucosamine, practical grade, catalogue number C-7170, Sigma Chem. Co., St. Louis, MO, U.S.A.). The hyphae of *G. clavigera* and *O. montium* used in this assay were collected from 250 mL liquid (malt extract broth) cultures, each of which was inoculated with a 5mm diam. agar plug from a stock culture and incubated on an orbital shaker (100 r.p.m., 20°C) for 7d. The hyphae were rinsed in distilled water, autoclaved, rinsed again, and dried prior to incorporation into the substrate assay. A set of nine controls containing only water was also inoculated. The flasks were secured at a random upright position on the platform of a rotary shaker. After 24 h incubation on the shaker (100 r.p.m.) at 20°C, bacterial populations were assessed as before with the Petroff- Hausser counting chamber and the microscope. The microscope (1000X) was also used to ob-

serve the degree of bacterial adhesion and aggregation on solids in the liquid cultures. The growth data were analyzed using analysis of variance; no statistical analysis of aggregation or adhesion was performed because there was no variance in the results.

Statistical analysis. Aside from FAME analysis, all statistical analyses were performed with Statistica 6.1 (Statsoft Inc., Tulsa, OK, U.S.A.). For data corresponding to larval sampling versus larval mortality, separate one-way analyses of variance (ANOVAs) were performed. Trunk aspect (degrees from North) was used as a categorical factor in one-way ANOVA, while site was nested as a random factor within moisture type for a two-factor ANOVA. In these and subsequent similar analyses, Levene's test was used to check for homogeneity of variance, and the Newman-Keuls multiple range test was used to compare means. Where Levene's test indicated significant ($P < 0.05$) heterogeneity of variance, ANOVA was performed after appropriate data transformation ($\arcsin\sqrt{Y}$). Where transformations were unable to remove significant ($P < 0.05$) heterogeneity of variance, non-parametric comparisons were employed. Larval mortality was subjected to regression analysis, using percent mortality as the dependant variable and either elevation or percentage of green trees attacked as independent variables. Student's t test for dependent samples was also used to compare hypothetical (expected) mortality values with mortality associated with the five most dominant bacteria. The expected values, derived from observed overall mortality rates, were generated by apportioning 50% of expected larval observations as dead, and 97% of expected adult observations as dead. For the experiment assessing fungal growth vs. bacterial inoculations, data were subjected to an ANOVA for nested factors. Bacterial isolates were treated as a random categorical variable nested within bacterial species, with five isolates per bacterial species, each replicated five-fold. Bacterial species was treated as a categorical variable nested within fungal species (3 per fungal species). A series of chi-square tests were utilized to compare the effect of chitinous substrates on the growth of *S. liquefaciens* and *R. aquatilis*, wherein the expected values were the mean growth in controls or mean growth in each of the substrates.

RESULTS

Isolation of bacteria from beetles. Characteristics of the sample sites are provided in Table 1. The slabs provided a total of 67 dead MPB adults, 2 living adults, 110 living larvae, and 112 dead larvae. All adults were mature (parental), and there were no exit holes apparent in the bark. A typical living larva is shown in Figure 2. Of the forty-four slabs containing insects, 1 lacked larvae and seven lacked adults. Larval mortality in the slabs ranged from 0 to 100%. Larval mortality at hydric sites (54.3%) was not significantly different from mortality in mesic sites (60.4), but mortality in the both of these site types was significantly greater (p =0.018) than mortality in the xeric sites (24.1%). There was also a significant (p = .039) effect of site location on mortality (Figure 3). There were no significant differences in mortality attributable to trunk aspect (p = 0.63). There were no significant regression trends for mortality versus elevation (p = 0.32) or mortality versus the percentage of green trees attacked (p = 0.686).

Identification of bacteria. Bacterial colonies were produced in 55% of the beetles sampled. This resulted in 161 pure cultures, of which 130 provided sufficient growth on TSBA medium for FAME analysis. Twenty-seven species of bacteria were identified in the beetles based on matches to reference strains in the MIDI TSBA40 library (Table 2). Eleven isolates had SI values less than 0.300, and were categorized as unidentified. Dendrogram cluster analysis separated the unidentified isolates into 5 species-related unknown groups based on

Figure 2. A typical living mountain pine beetle larva included in the cultural isolations.

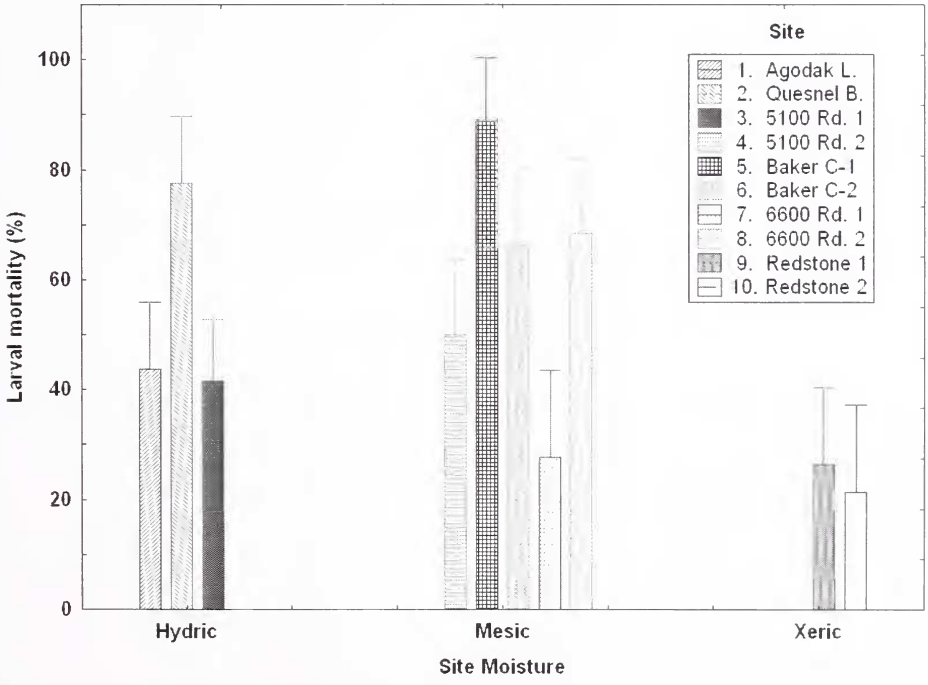

Figure 3. The effect of site location and moisture on the mortality of 232 mountain pine beetle larvae in the Southern Interior of B.C., March, 2003. Site numbers correspond to the sites listed in Table 1.

their FAME profiles. Five of six isolates in one of the unidentified species groups were found in dead larvae. *Serratia* spp. were frequently isolated from larvae (living and dead), possibly indicating a role in the insect's normal gut microflora. *R. aquatilis* was also frequently isolated (3 adults, 9 larvae). The incidence of *R. aquatilis* was widespread, occurring at eight of the ten sites and in 11% of the insects, but it was mainly (93%) found in larval cadavers. Other bacteria occurring principally in dead insects were less frequently isolated, although mortality associated with *Pseudomonas syringae* var. *tabaci* (Wolf & Foster) Young et al. was significant ($p = 0.01$) in comparison to expected mortality (Table 2). Twelve bacterial species were isolated from

Table 2.
The incidence of bacterial species isolated from mountain pine beetle, and the mortality of associated adults and larvae.

Species	Similarity index (%)[1]	Incidence (%)[2]	Isolates in living (and dead) larvae (no.)[3]	Isolates in living (and dead) adults (no.)[3]	Total sites (no.)	Beetle mortality (%)[4]
Serratia liquefaciens	75 ± 8	22	11 (10)	0 (7)	8	61
Serratia plymuthica	76 ± 8	17	7 (8)	1 (6)	7	62
Rahnella aquatilis	89 ± 5	11	1 (10)	0 (3)	8	93 *
Pseudomonas syringae v. *tabaci*	91 ± 2	5	0 (4)	0 (2)	3	86 *
Hafnia alvei	79 ± 6	5	1 (3)	0 (2)	4	83
Pantoea citrea	76 ± 4	4	1 (2)	0 (2)	4	60
Enterobacter pyrinus	71 ± 5	3	2 (2)	0 (0)	3	50
Erwinia chrysanthemi biotype IV/VI	75 ± 9	3	0 (0)	0 (4)	3	100
Proteus vulgaris GC supgp. A	74 ± 7	2	1 (0)	0 (1)	2	60
Kluyvera ascorbata	62 ± 8	2	1 (0)	0 (2)	2	67
Brevibacillus agri	86 ± 0	2	0 (3)	0 (0)	2	100
Pseudomonas syringae v. *phaseolicola*	91 ± 2	2	2 (1)	0 (0)	1	33
Paenibacillus lentimorbus	64 ± 1	2	1 (1)	0 (0)	1	50
Salmonella typhimurium GC subgp. B	72 ± 13	2	2 (0)	0 (0)	2	0
Sphingobacterium multivorum	84 ± 6	2	0 (1)	0 (1)	1	100
Kluyvera intermedia	73	1	0 (0)	0 (1)	1	100
Pseudomonas fluorescens biotype G/C	92	1	0 (1)	0 (0)	1	100
Serratia grimesii Grimont	81	1	0 (1)	0 (0)	1	50
Bacillus megaterium subgp. A	92	1	0 (1)	0 (0)	1	100
Cedecea davisae	65	1	0 (1)	0 (0)	1	100
Chromobacterium violaceum	89	1	0 (0)	0 (1)	1	100
Pantoea agglomerans GC subgp. 1	75	1	0 (1)	0 (0)	1	100
Klebsiella pneumoniae var. *ozaenae*	85	1	0 (1)	0 (0)	1	100
Raoultella terrigena	59	1	0 (0)	0 (1)	1	100
Pseudomonas putida biotype B	85	1	0 (1)	0 (0)	1	100
Salmonella cholerasuis v. *arizonae*	67	1	1 (0)	0 (0)	1	0
Sphingomonas paucimobilis	97	1	0 (1)	0 (0)	1	100
Xenorhabadus nematophilus	71	1	0 (0)	0 (1)	1	100

[1] Where there was more than one observation, indices in this column are shown as the mean ± s.d.
[2] The incidence percentage is calculated as (number of beetle hosts / number of beetles tested) x 100.
[3] In this column, the number of isolates found in living insects is followed by the number of isolates found in dead insects, in parentheses.
[4] Percentages followed by an asterisk are significantly different ($p < 0.01$, Student's t test for dependent samples) than expected mortality (larvae = 51%; adults = 100%).

bark samples also producing insects with *R. aquatilis*; these included three isolates of *P. syringae* var. *tabaci* (Table 3).

Bacterial inhibition of bluestain fungi. There were significant differences in growth inhibition for parameters corresponding to fungal species ($F_1 = 107.4$, $p = 0.000000$) and bacterial species ($F_2 = 3.2$, $p = 0.045270$). Bacterial isolates within species had no significant impacts ($F_{12} = 1.5$, $p = 0.125199$). Bacterial inhibition of *O. montium* was nearly twice the inhibition experienced by *G. clavigera*. Bacterial species produced similar levels of inhibition against the growth of *O. montium*. *S. liquefaciens* was significantly ($p < 0.05$) less inhibitory towards the growth of *G. clavigera* (Table 4). Caution is warranted in quantitative interpretation of these results, in that volatile compounds from the microbes could permeate throughout the plates, affecting the scale of the response in both controls and treatments. The relative responses, however, indicate a potential for inhibition.

Interactions between bacteria and chitin-containing substrates. Chi-square comparisons indicated that *S. liquefaciens* and *R. aquatilis* thrived on autoclaved mycelium and, to a greater degree, on practical grade chitin (Table 5). All cultures with combined inocula appeared to have approximately equal proportions of each bacterial species when cell morphology was observed under a compound microscope. Combined cultures generally performed as well as individual cultures, with no clear indication of a competitive advantage for either of the two bacteria. The bacteria did not adhere to the smooth surfaces of the autoclaved mycelia, but *R. aquatilis* would occasionally adhere to minute rough areas on particles of the practical grade chitin. These rough areas appeared to serve as loci for adhesion and aggregation of the bacteria. When *R. aquatilis* was combined with *S. liquefaciens*, bacteria adhered to many more sites on the chitin particles, and numerous small bacterial aggregates formed (Table 5).

DISCUSSION

Although it was not isolated from every dead larva, *R. aquatilis* appears to be associated with mortality in MPB. Parental adults had an expected mortality of 100% during the winter; *R. aquatilils* is therefore not expected to have caused any increased mortality in adults. *R. aquatilis* is an enteric bacterial species that occurs widely in water and soil environments (Berge *et al.* 1991, Heulin *et al.* 1994, Horie *et al.* 1985), and it has been detected from a variety of insects, including bark beetles (Vasanthakumar *et al.* 2006; Delalibera *et al.* 2005). There are reports of this species linked to opportunistic bacterial infections in humans (Caroff *et al.* 1998, Lebessi *et al.* 1990, Maraki *et al.* 1994, Matsukura *et al.* 1996, Oh and Tay 1995), and some strains are reported to possess antagonistic properties against bacterial plant diseases (Laux *et al.* 2002, 2003). Regarding quantification, the frequencies reported here are for surface-sterilized insects receiving a moderate amount of compression during placement on agar. This was sufficient to express hindgut contents onto the isolation medium, but a specific method for extraction of gut contents could have generated higher incidence statistics for bacteria tending to aggregate inside the larvae. Using other isolation methods, for example different isolation media or serial dilutions of ground tissue, would also yield other non-dominant bacteria and a greater diversity of species. The results thus pertain to the proportion of insects where the particular bacterial species are dominant, rather than quantifying the actual proportion of insects associated with the bacteria.

Some genotypic and phenotypic heterogeneity is reported for *R. aquatilis* (Brenner *et al.* 1998, Pokhil 1998, Selenska-Pobell *et al.* 1995, Varbanets *et al.* 2004); the biological role of isolates found in MPB remains an open question. For example, further study would be needed to determine whether the bacterium is pathogenic, or

Table 3.
Bacterial incidence in living and dead larvae of Mountain Pine Beetle, where larvae in the same bark sample were also infested with *Rahnella aquatilis*.

Species	Class	Order	Isolates	Site(s)[1]
Paenibacillus lentimorbus	Bacilli	Bacillales	2	3
Pantoea agglomerans GC subgroup	Gammaproteobacteria	Enterobacteriales	1	8
Enterobacter pyrinus	Gammaproteobacteria	Enterobacteriales	1	6
Erwinia chrysanthemi biotype VI	Gammaproteobacteria	Enterobacteriales	3	5, 6, 10
Hafnia alvei	Gammaproteobacteria	Enterobacteriales	1	10
Proteus vulgaris GC subgroup	Gammaproteobacteria	Enterobacteriales	1	6
Pantoea citrea	Gammaproteobacteria	Enterobacteriales	1	3
Pseudomonas fluorescens biotype G	Gammaproteobacteria	Pseudomonadales	1	10
Pseudomonas syrningae var. *tabaci*	Gammaproteobacteria	Pseudomonadales	3	6
Serratia liquefaciens	Gammaproteobacteria	Enterobacteriales	5	3, 6, 8
Serratia plymuthica	Gammaproteobacteria	Enterobacteriales	7	3, 6, 8
Sphingomonas paucimobilis	Alphaproteobacteria	Sphingomonadales	1	5

[1] Original collection sites listed in Table 1

Table 4.
The effect of bacterial species with fungal species assayed for growth inhibition.

Bluestain Fungus	Bacterial species	% Inhibition of fungal growth[1]
O. montium	*R. aquatilis*	74.1 a
	S. liquefaciens	73.2 a
	S. plymuthica	68.2 a
G. clavigera	*R. aquatilis*	46.0 bc
	S. liquefaciens	33.7 c
	S. plymuthica	46.2 bc

[1] Means in this column followed by the same letter are not significantly different according to the Newman-Keuls multiple range test ($p > 0.05$). The test was performed on transformed data (arcsin$\sqrt{Y}$); actual %Inhibition is shown.

simply an opportunist that flourishes after the larvae succumb to viruses, nematodes, or other stresses not screened in this study.

The three most dominant bacteria inhibited the growth of the bluestain fungi. None of the bacterial species were significantly more inhibitory versus either bluestain fungal species. None of the bacterial species aggregated on autoclaved mycelia of the fungi. These results are interesting, in that bacteria are thought to mediate or inhibit the growth of fungi associated with two other *Dendroctonus* spp.: *D. frontalis* Zimmermann (southern pine beetle) and *D. rufipennis* (Kirby) (spruce beetle) (Cardoza *et al.* 2006, Scott *et al.* 2008). Further tests

Table 5.
The effect of chitin-containing substrates on the growth, adhesion and aggregation of *Serratia liquefaciens* and *Rahnella aquatilis*.

Substrate	*S. liquefaciens*[1]	*R. aquatilis*[1]	Density (10^6 bacteria mL^{-1})[2]	Adhesive cultures (no.)	Aggregating cultures (no.)
None	+		0.36 a	0	0
		+	0.32 ac	0	0
	+	+	0.19 a	0	0
G. clavigera	+		5.24 bc	0	0
(autoclaved hyphae)		+	4.06 bc	0	0
	+	+	5.22 bc	0	0
O. montium	+		7.88 c	0	0
(autoclaved hyphae)		+	6.09 bc	0	0
	+	+	8.23 c	0	0
Crab shell	+		10.44 d	0	0
		+	15.34 d	3	0
	+	+	16.69 d	3	3

[1] A plus sign (+) in these columns indicates cultures were inoculated with the species listed in the column heading.
[2] Means in this column followed by the letter 'a' are not significantly different from the expected control concentration of 0.29×10^6 bacteria mL^{-1} using the Chi-square test ($p > 0.05$). Means followed by the letter 'b' are not significantly different from the expected concentration for growth on hyphae of *G. clavigera* (4.77×10^6 bacteria mL^{-1}), means followed by the letter 'c' are not significantly different from the expected concentration for growth on hyphae of *O. montium* (7.73×10^6 bacteria mL^{-1}), and means followed by the letter 'd' are not significantly different from the expected concentration for growth on Crab shell ($15.167.73 \times 10^6$ bacteria mL^{-1}), also using Chi-square tests ($p > 0.05$).

would be necessary to understand the cause of the inhibition. Nutrient depletion, pH changes, or various secondary compounds are all examples of possible inhibitory factors. Although *R. aquatilis* is reported to grow well on wood (Kallioinen *et al.* 2003), proliferation of bluestain fungi in the beetle galleries suggests that the enteric bacteria primarily inhabit the insect gut, where they would have limited impact on the spread of the fungi. Another study has shown that the growth of *O. montium* is stimulated by microbes isolated from the galleries of MPB, including three yeasts (*Candida* sp., *Pichia scolyti*, and an unidentified basidiomycete) and a bacterial species (*Micrococcus* sp.). However, the same study also found that *Candida* sp., the basidiomycete yeast, and *Micrococcus* sp. were inhibitory to the growth of *G. clavigera*. Isolated from fresh phloem near MPB attacks, *Bacillus pumilus* is also reported to be inhibitory to the growth of both fungi (Adams *et al.* 2008). There is probably a diverse range of antagonistic and mutualistic interactions among the various microbes that associate with MPB.

The adherence and aggregation of *R. aquatilis* and *Serratia* spp. on arthropod chitin could indicate a role for microbial consortia in the observed beetle mortality. Insect hindguts can possess chitinous structures that facilitate digestion through bacterial aggregation (Hackstein and Stumm 1994). *R. aquatilis* and *Serratia* spp. are involved in biofilm formation (Steidle *et al.* 2001). Further research is needed to understand the prevalence of *R. aquatilis*/*Serratia*

spp. consortia in the environment and how *R. aquatilis* becomes established in MPB populations. The abundant growth of *S. liquefaciens* and *S. plymuthica* on autoclaved mycelium of *Ophiostoma* spp. agreed with the reported chitinolytic properties of these species (Berg *et al.* 1999, Joshi *et al.* 1988). Presumably, larval MPB could benefit from any digestive action of these bacteria on bluestain fungi. However, other bacterial species could also persist in the *R. aquatilis*/*S. liquefaciens* consortium, potentially creating chronic stress in the insect host or affecting cold-tolerance. In this study, slabs producing specimens with *R. aquatilis* also produced specimens containing *Pseudomonas* spp., an ice-nucleating species (Lee *et al.* 1998).

The association of *Serratia* spp. with living beetles agrees with a previous study of *D. frontalis*, where bacteria isolated from the gut of healthy insects included *Serratia* spp. (Moore, 1971). Earlier research on southern pine beetle correlated the occurrence of several bacterial species with beetle mortality, including *Serratia marcescens* Bizio, *Pseudomonas aeruginosa* (Schroeter) Migula, *Pseudomonas fluorescens* Migula, *Bacillus thuringiensis* Berliner, *Bacillus cereus* Frankland & Frankland, and *Flavobacterium* sp. (Moore 1971). In further tests on southern pine beetle, pathogenicity was demonstrated for *S. marcescens*, the *Pseudomonas* spp., and the *Bacillus* spp. (Moore 1972b).

Without further *in vivo* testing, it is difficult to assign a definite pathogenic or opportunistic role for *R. aquatilis* in larvae or adults of MPB. A pathogenic form of *R. aquatilis* might have utility as a biological control agent, since the species is motile and could potentially penetrate beetle galleries during wet weather. On the other hand, even if *R. aquatilis* is simply an opportunist that aggregates with species such as *S. liquefaciens*, this mechanism of action might still be exploited to reduce the cold tolerance of the insect. Answers are needed regarding the natural frequency of particular bacterial associations, but the deliberate introduction of aggregating bacteria along with ice-nucleating species could reduce the cold tolerance of MPB. This could be especially beneficial if climate warming continues to exacerbate the beetle impacts. In any eventual effort to develop biological control agents, the occurrence and influence of other pathogens in larvae (viruses, nematodes, etc.) should also be explored. The geographic occurrence of *R. aquatilis* should also be surveyed and its mode of distribution within brood trees elucidated. A more complete understanding of microbial influences and constraints on MPB may help us deal with future outbreaks.

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